Form PTO-1449 (modified)	Atty. Docket No. 4001.002200	Serial No. 09/351,543
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	**Applicant** Philip E. Thorpe and Sophia Ran	
	Filing Date: July 12, 1999	**Unknown**

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 1*	*See Page 2-5*

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Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date of App.
	A1	5,855,866	01.05.99	Thorpe *et al.*	424	1.49	
	A2	5,863,538	01.26.99	Thorpe *et al.*	424	136.1	
	A3	5,776,427	07.07.98	Thorpe *et al.*	424	1.49	
	A4	5,767,298	06.16.98	Daleke	554	80	
	A5	5,660,827	08.26.97	Thorpe *et al.*	424	152.1	
	A6	5,658,877	08.19.97	Tsao	514	2	
	A7	5,632,986	05.27.97	Tait *et al.*	424	94.64	
	A8	5,627,036	05.06.97	Reutelingsperger	435	7.21	
	A9	5,344,758	09.06.94	Krilis *et al.*	433	4.1	
	A10	5,296,467	03.22.94	Reutelingsperger	514	12	

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	B1	WO 98/04294	February 5, 1998	PCT			
	B2	WO 97/17084	May 15, 1997	PCT			
	B3	WO 96/17618	June 13, 1996	PCT			
	B4	WO 96/01653	January 25, 1996	PCT			
	B5	WO 95/34315	December 21, 1995	PCT			
	B6	WO 95/27903	October 19, 1995	PCT			
	B7	WO 95/19791	July 27, 1995	PCT			
	B8	WO 93/17715	September 16, 1993	PCT			
	B9	WO 91/07187	May 30, 1991	PCT			

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	C8	Denekamp, "Vascular Attack as a Therapeutic Strategy for Cancer," *Cancer and Metastasis Reviews*, 9:267-282, 1990.
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	C34	Co-pending Application Serial No. 09/351,862; Entitled: "Cancer Treatment Kits Using Antibodies to Aminophospholipids"; filed July 12, 1999 (Attorney Docket Nos. 4001.002282, UTSD:549--1).
	C35	Co-pending Application Serial No. 09/351,457; Entitled: "Cancer Treatment Methods Using Therapeutic Conjugates that Bind to Aminophospholipids"; filed July 12, 1999 (Attorney Docket Nos. 4001.002300, UTSD:556).
	C36	Co-pending Application Serial No. 09/351,598; Entitled: "Cancer Treatment Compositions Comprising Therapeutic Conjugates that Bind to Aminophospholipids"; filed July 12, 1999 (Attorney Docket Nos. 4001.002382, UTSD:556--1).

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	Filing Date: July 12, 1999	**Unknown**

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 1*	*See Page 2-5*

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List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant: Philip E. Thorpe and Sophia Ran	
	Filing Date: July 12, 1999	Group: 1614

U.S. Patent Documents	Foreign Patent Documents	Other Art
	See Page 1	*See Page 1*

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	B10	WO 98/43678	Oct. 8, 1998	PCT			
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	C38	Diaz *et al.*; "Synthesis of Disulfide-Containing Phospholipid Analogs for the Preparation of Head Group-Specific Lipid Antigens: Generation of Phosphatidylserine Antibodies;" *Bioconjugate Chem.*; 9:250-254, 1998.
	C39	Kasina *et al.*; "Preformed Chelate TC-99m Radiolabeling of r-Annexin V for Arterial Thrombus Imaging;" *Nucl. Med.*; Vol. 37. No. 5, 29P.
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	C41	Sarrot-Reynauld and Massot; "Antiphospholipid Antibodies Paraneoplastic Syndrome Revealing Prostatic Cancer;" *Lupus*; Vol. 5, No. 5, pp 528, 1996.
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	C44	International Search Report for PCT/US99/15668, mailed December 29, 1999

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U.S. Patent Documents	Foreign Patent Documents	Other Art *See Page 1*

U.S. Patent Documents

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	C45	Ferro *et al.*, "Coexistence of Anti-Phospholipid Antibodies and Endothelial Perturbation in Systemic Lupus Erythematosus Patients with Ongoing Prothrombotic State," *Circulation*, 95(6);1425-1432, 1997.
	C46	Fishman *et al.*, "Autoimmunity and Cancer - Beneficial Relationships: a new Concept for the Production of Human Monoclonal Antibodies (Review)," *International Journal of Oncology*, 10:901-904, 1997.
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	C48	International Search Report for PCT/US99/15600, mailed January 26, 2000.

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U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page*	*See Page*

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	A11	6,051,230	04.18.00	Thorpe *et al.*	424	178.1	
	A12	6,036,955	03.14.00	Thorpe *et al.*	424	136.1	
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U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page*	*See Page*

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	A17	6,093,399	July 25, 2000	Thorpe and Ran	424	182	

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U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page*	*See Page 1*

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U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page*	*See Page*

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See Page 1	*See Page*	*See Page*

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See Page	*See Page 1*	*See Page*

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July 24, 2002
Date

Shelley P.M. Fussey

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Thorpe and Ran

Serial No.: 09/998,833

Filed: November 30, 2001

For: COMBINED CANCER TREATMENT METHODS USING ANTIBODIES TO AMINOPHOSPHOLIPIDS

Group Art Unit: 1642

Examiner: Bansal, G.

Atty. Dkt. No.: 4001.002299

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In compliance with the duty of disclosure under 37 C.F.R. § 1.56, it is respectfully requested that this Supplemental Information Disclosure Statement be entered and the documents listed on attached Form PTO-1449 be considered by the Examiner and made of record in the present case. Copies of the listed documents required by 37 C.F.R. § 1.98(a)(2) are enclosed for the convenience of the Examiner.

In accordance with 37 C.F.R §§ 1.97(g),(h), this Supplemental Information Disclosure Statement is not to be construed as a representation that a search has been made, and is not to be construed to be an admission that the information cited is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

The present Supplemental Information Disclosure Statement is being filed prior to the receipt of a first Official Action reflecting an examination on the merits, and hence is timely filed in accordance with 37 C.F.R. § 1.97(b). Therefore, no fees should be required. Even if an Official Action had been issued in the last few days, no fees would be required in light of the following information.

In accordance with 37 C.F.R. § 1.97(e)(2), it is hereby certified that the documents listed in the accompanying Form PTO-1449 were not cited in a communication from a foreign patent office in a counterpart foreign application. The listed documents were cited in an Official Action on the merits in a co-pending application of the present inventors, Serial No. 09/351,862 (Attorney Docket No. 3999.002399), mailed from the P.T.O. on July 15, 2002, less than three months before the filing of the present statement, and evidently could not have been submitted before receipt.

In accordance with 37 C.F.R. § 1.98 (a)(3), a concise explanation of the relevance of the submitted André article, as it is presently understood, is supplied. The following concise explanation is the Abstract for André taken from the reference:

"**Summary - Tumoral angiogenesis: physiopathology, prognostic value and therapeutical prospects.**

Introduction. - Angiogenesis activation plays a crucial role in tumoral growth and metastases dissemination. This review summarizes and analyzes current knowledge on molecular mechanisms related to angiogenesis and the prognostic value of its effectors. It also focuses on the therapeutical relevance of various drugs that might inhibit angiogenesic processed.

Current knowledge and key points. - Tumor angiogenesis involves complex interactions between tumoral, stromal, endothelial cells, fibroblasts and the extracellular matrix. Normal and malignant angiogenesis depends on the balance of proangiogenic and antiangiogenic factors. Endothelial cells are activated by growth factors, such as Vascular Endothelial Growth Factor (VEGF), and proliferate; they release proteases able to induce degradation of the basement membrane and extracellular matrix, and undergo migration and tubulogenesis. Angiostatin and endostatin are two powerful inhibitors of angiogenesis in experimental models. Assessment of intratumoral microvessel density and quantification of angiogenic factors, including VEGF, are of prognostic value in most cancers, particular in breast cancer. However, the use of these prognosis markers in clinical practice is still controversial due to the lack of prospective studies and to technical limits inherent to the scoring and standardization of immunohistochemical methods.

Future, prospects and projects. - Better understanding of the molecular basis of angiogenesis allows the development of new therapeutical strategies. Biochemical targets of antiangiogenic therapy are: the interaction between angiogenic factors and their receptors; the interaction of endothelial cells with the extracellular matrix; and intracellular signaling pathways. Angiogenesis inhibitors may not cause tumor regression, but inhibit cellular growth and produce "disease dormancy". Extensive phase I to III clinical trials involving antiangiogenesis therapy are in progress."

No fees should be due in connection with the filing of this Supplemental Information Disclosure Statement. However, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be deemed necessary for any reason relating to these materials, the Examiner should contact the undersigned representative to discuss deduction from Williams, Morgan & Amerson Deposit Account No. 50-0786/4001.002299.

Respectfully submitted,

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Form PTO-1449 (modified)	Atty. Docket No. 4001.002299	Serial No. 09/998,833
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant Philip E. Thorpe and Sophia Ran	
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U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page	*See Page*	*See Page 1*

U.S. Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date of App.

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C56	André *et al.*, "Angiogenese Tumorale: Physiopathologic, Valeur Prognostique et Perspectives Therapeutiques", *Rev. Med. Interne.*, 19:904913, 1998.
	C57	Thiagarajan and Benedict, "Inhibition of Arterial Thrombosis by Recombinant Annexin V in a Rabbit Carotid Artery Injury Model" *Circulation*, 96(7):2339-47, 1997.

EXAMINER:	DATE CONSIDERED:

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Rev Méd Interne 1998 ; 19 : 904-13

Mise au point

Angiogenèse tumorale : physiopathologie, valeur pronostique et perspectives thérapeutiques

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(Reçu le 27 avril 1998 ; accepté le 10 juillet 1998)

Résumé

Propos. – Dans les cancers, l'activation de l'angiogenèse joue un rôle important dans le développement tumoral et dans la dissémination métastatique. L'objectif de cette revue est de faire le point sur la physiopathologie et la valeur pronostique de l'angiogenèse tumorale, et sur les perspectives thérapeutiques visant à l'inhiber.

Actualités et points forts. – L'angiogenèse tumorale se met en place grâce à des interactions cellulaires et moléculaires complexes entre cellules cancéreuses, cellules endothéliales, cellules du stroma tumoral et les constituants de la matrice extracellulaire. La croissance et la dissémination des tumeurs solides malignes dépendent d'un équilibre existant entre régulateurs positifs et négatifs de l'angiogenèse tumorale. Les cellules endothéliales peuvent, grâce à l'action de facteurs de croissance sécrétés par les cellules cancéreuses eg *vascular endothelial growth factor* (VEGF), entrer dans le cycle cellulaire, migrer après rupture de la membrane basale et s'organiser spatialement, pour former les vaisseaux irriguant la tumeur. L'angiostatine et l'endostatine, sont deux puissants inhibiteurs de l'angiogenèse dans des modèles de carcinogenèse expérimentale. De nombreuses études rétrospectives, portant le plus souvent sur la densité en microvaisseaux et plus rarement sur le VEGF, ont montré dans de nombreux cancers et en particulier le cancer du sein, une association entre angiogenèse et pronostic. Cependant, l'intérêt en clinique de ces facteurs pronostiques est controversé en raison du manque d'études prospectives et des limitations inhérentes à la quantification et à la standardisation des études immunohistochimiques.

Perspectives et projets. – Une meilleure compréhension des processus moléculaires et physiopathologiques activant l'angiogenèse tumorale permet d'élaborer de nouvelles approches thérapeutiques. Actuellement, les inhibiteurs de l'angiogenèse visent trois cibles moléculaires : la liaison des facteurs angiogéniques aux récepteurs, l'interaction entre les cellules endothéliales et la matrice extracellulaire et la transmission intracellulaire du signal. Les thérapeutiques anti-angiogéniques sont en cours d'évaluation dans des études de phase I à III.

angiogenèse / cancer / *vascular endothelial growth factor* / signalisation

Summary – Tumoral angiogenesis: physiopathology, prognostic value and therapeutical prospects.

Introduction. – *Angiogenesis activation plays a crucial role in tumoral growth and metastases dissemination. This review summarizes and analyzes current knowledge on molecular mechanisms related to angiogenesis and the prognostic value of its effectors. It also focuses on the therapeutical relevance of various drugs that might inhibit angiogenesic processes.*

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Future prospects and projects. – *Better understanding of the molecular basis of angiogenesis allows the development of new therapeutical strategies. Biochemical targets of antiangiogenic therapy are: the interaction between angiogenic factors and their receptors; the interaction of endothelial cells with the extracellular matrix; and intracellular signaling pathways. Angiogenesis inhibitors may not cause tumor regression, but inhibit cellular growth and produce « disease dormancy ». Extensive phase I to III clinical trials involving antiangiogenesis therapy are in progress.*

angiogenesis / cancer / vascular endothelial growth factor / signal transduction

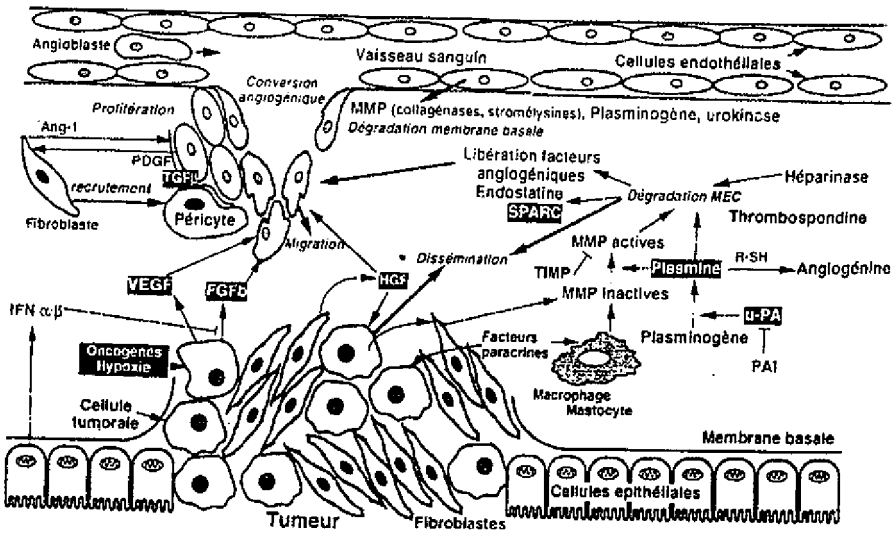

Fig 1. Interactions entre cellules endothéliales, cellules tumorales et stromales pendant l'angiogenèse et l'invasion tumorale. L'angiogenèse fait intervenir : 1) la secrétion et l'activation en cascade de métalloprotéinases et de sérine protéases (plasminogène, urokinase) par les cellules endothéliales ; 2) la dégradation de la membrane basale et de la matrice extracellulaire ; 3) la prolifération et la migration des cellules endothéliales ; 4) la reconstitution de la paroi vasculaire après assemblage de la membrane basale et la conversion de cellules stromales en péricytes. Ces différentes étapes sont sous le contrôle d'effecteurs positifs (encre blanche/fond noir), qui sont des facteurs paracrines : 1) sécrétés par les cellules tumorales (VEGF, bFGF), consécutivement à l'hypoxie ou à la dérégulation de voies oncogéniques (p21-ras, p53) ; 2) libérés de la matrice extracellulaire consécutivement à l'action de protéases, d'héparinases ou des fragment de protéolyse de la matrice extracellulaire elle-même (SPARC) ; 3) produits par les fibroblastes du stroma tumoral. Un contrôle négatif est exercé par : 1) d'autres facteurs paracrines, l'interféron α/β (IFN) sécrété par les cellules épithéliales (inhibition de l'expression du bFGF) ; 2) les inhibiteurs des métalloprotéases matricielles (MMP) : TIMP, un fragment de la plasmine généré en présence de radicaux souffrés, R-SH (angiogénine) ; 3) des constituants de la matrice extracellulaire ou certains de leurs fragments : thrombospondine, collagène XVIII (endostatine). La présence d'angioblastes dans la circulation sanguine pourrait également contribuer à la néovascularisation tumorale.

La vasculogenèse est la formation de vaisseaux à partir des précurseurs des cellules endothéliales, les angioblastes, qui proviennent du mésoderme pendant l'embryogenèse. L'angiogenèse est la formation et le branchement de nouveaux vaisseaux à partir de vaisseaux préexistants (migration et multiplication des cellules endothéliales).

Approximativement, 1 à 6×10^{13} cellules endothéliales forment les vaisseaux sanguins chez un adulte, représentant un poids d'environ 1 kg et couvrant une surface d'environ 1 000 m^2. Le temps de renouvellement de ces cellules, normalement quiescentes, est de 1 000 jours. Les cellules endothéliales sont, après les cellules nerveuses, les cellules dont la demi-vie est la plus longue. Seulement 0,01 % des cellules endothéliales résidentes est engagé dans le cycle de division cellulaire (phase proliférative). Cependant, durant l'angiogenèse, les cellules endothéliales des capillaires peuvent proliférer aussi rapidement que les cellules de la moelle osseuse, qui ont un temps de renouvellement de 5 jours. Sous l'effet de stimulations par des facteurs angiogéniques, les cellules endothéliales peuvent entrer dans le cycle cellulaire et dégrader la membrane basale. Elles peuvent s'organiser en capillaires dans le stroma périvasculaire, induire la différenciation de cellules mésenchymateuses en péricytes pour former de nouveaux vaisseaux fonctionnels adaptés à leur microenvironnement tissulaire. Un ensemble de processus biochimiques est impliqué à chaque étape, incluant la production de protéases (collagénases, urokinase) par les cellules tumorales, les fibroblastes et les cellules endothéliales *(figure 1)*. Le développement et la croissance des cellules endothéliales sont régulés par des facteurs pro- et anti-angiogéniques. La néovascularisation est un processus clé du développement embryonnaire, des fonctions reproductrices (menstruation, formation du corps jaune, formation du placenta) et de la réparation tissulaire lors de traumatismes (cicatrisation) et d'ischémies. L'angiogenèse peut cependant devenir pathologique, et contribuer au développement de certaines maladies comme les rétinopathies diabétiques, la polyarthrite rhumatoïde, le psoriasis, les hémangiomes de l'enfant et la progression tumorale [1]. L'angiogenèse tumorale est un processus clé pour la croissance et la dissémination des tumeurs solides malignes et des leucoses. Il est actuellement admis

qu'une tumeur de diamètre supérieur ou égal à 3 mm a besoin de néovaisseaux pour lui apporter oxygène, nutriments et éviter l'hypoxie et l'apoptose des cellules tumorales [2, 3].

Des fragments tumoraux, implantés dans des territoires avasculaires comme la cornée, sont vascularisés en quelques jours par des néovaisseaux. La tumeur peut donc assurer sa propre vascularisation en relarguant vers les vaisseaux préexistants des médiateurs solubles qui déclenchent la conversion du phénotype quiescent des cellules endothéliales en phénotype angiogénique. Chez des patients atteints de cancer, les micrométastases sont souvent asymptomatiques et cliniquement non détectables pendant des mois ou des années. La croissance d'une tumeur primitive et de ses métastases dépend de l'équilibre existant entre les régulateurs positifs et négatifs de l'angiogenèse. Si les activateurs prédominent, la tumeur et ses métastases peuvent se développer. Si les inhibiteurs prédominent, la tumeur et ses métastases ne peuvent croître au-delà d'un volume de quelques mm^3, l'index apoptotique étant élevé en l'absence d'angiogenèse [4, 5]. Sur plusieurs modèles de souris transgéniques, présentant une surexpression ou une invalidation *(knockout)* d'un ou de plusieurs gènes impliqués dans l'angiogenèse, on peut vérifier que l'activation de l'angiogenèse se produit effectivement à un stade précoce du développement tumoral. L'induction de l'angiogenèse sur ces modèles constitue une étape indépendante et limitante du développement des tumeurs [6]. L'angiogenèse se met en place à la périphérie de la tumeur dans le tissu conjonctif. L'arrivée de néovaisseaux au contact de la membrane basale peut être observée à un stade de dysplasie ou de carcinome in situ avant l'émergence d'une tumeur invasive, suggérant que l'activation de l'angiogenèse soit un phénomène progressif et précoce [4, 7]. L'angiogenèse, établie pendant les stades précancéreux, a été étudiée dans le sein et le col de l'utérus par immunohistochimie (anticorps anti-facteur Willebrand et anti-CD31). Dans ces tumeurs, la néoangiogenèse tumorale est activée et commence à se mettre en place dès le stade de carcinome in situ [4].

L'angiogenèse tumorale implique des interactions cellulaires et moléculaires complexes entre les cellules cancéreuses, les cellules endothéliales, les cellules du stroma tumoral et péritumoral, et les constituants de la matrice extracellulaire. L'activation des cellules endothéliales peut se faire par sécrétion de facteurs angiogéniques:
– soit de façon directe par les cellules cancéreuses elles-mêmes par sécrétion de *vascular endothelial growth factor* (VEGF) en réponse à l'hypoxie [8, 9] ou comme conséquence de l'activation de certains oncogènes ou de certains gènes supresseurs de tumeur comme *ras* et *p53*;
– soit de façon indirecte par d'autres types cellulaires présents dans le stroma tumoral (macrophages, mastocytes, fibroblastes, cellules dendritiques) « recrutés » par les cellules cancéreuses;
– soit par certains composants et fragments protéolytiques de la matrice extracellulaire ou le relargage de facteurs angiogéniques séquestrés par les protéoglycanes [7].

Tableau I. Régulateurs endogènes de l'angiogenèse tumorale.

Facteurs proangiogéniques
- Angiogenine
- Angiopoïétine-1
- *Epidermal growth factor* (EGF)
- *Fibroblast growth factor* (FGF) acide et basique
- *Hepatocyte growth factor* (HGF)
- Interleukine-8 (IL-8)
- *Placenta growth factor*
- *Platelet-derived growth factor* (PDGF)
- *Secreted protein acidic and rich in cystein* (SPARC)
- *Transforming growth factor* (TGF) α et β*
- *Tumor necrosis factor* (TNF) α
- *Vascular endothelial growth factor* (VEGF)
- VEGF-B, VEGF-C et VEGF-D

Facteurs anti-angiogéniques
- Angiostatine
- Angiopoïétine-2
- Endostatine
- Facteur 4 plaquettaire
- Fragments de la prolactine
- Fragment de la matrix metalloprotease 2 (MMP2)
- Inhibiteurs tissulaires des métalloprotéinases (TIMP)
- Interférons α et β
- Protamine
- *Transforming growth factor* (TGF) β*
- Trombospondine-1

*Rôle complexe du TBF β, permissif ou inhibiteur selon le modèle étudié. Il inhibe in vitro la croissance des cellules endothéliales et induit in vivo l'angiogenèse.

De nombreux médiateurs *(tableau I)* exercent une action proangiogénique ou anti-angiogénique. Certains de ces facteurs, comme le VEGF, se fixent aux récepteurs membranaires à activité tyrosine kinase spécifiques localisés sur les cellules endothéliales et déclenchent la signalisation intracellulaire. Une meilleure connaissance des mécanismes qui régulent l'invasion et l'angiogenèse permet d'envisager des traitements à visée anti-angiogénique actuellement en développement. Ces traitements représentent l'un des axes de recherche majeurs en cancérologie.

FACTEURS ANGIOGÉNIQUES

Vascular endothelial growth factor (VEGF) et ses peptides apparentés

Parmi les nombreux médiateurs intervenant dans l'angiogenèse, le premier facteur de croissance spécifique

de la cellule endothéliale, le VEGF, a été identifié en 1989 [10]. Les études réalisées depuis ont montré l'importance du VEGF dans les processus angiogéniques normaux et pathologiques. Le clonage moléculaire et la caractérisation du VEGF ont été réalisés simultanément par trois équipes [11-13]. Ce facteur est une glycoprotéine de 40-45 kDa, liant l'héparine, dont la séquence présente de faibles homologies avec les chaînes A et B du facteur de croissance plaquettaire (PDGF). Le gène codant le VEGF humain, localisé sur le chromosome 6, est constitué de huit exons [10]. Le VEGF est exprimé très précocement pendant l'embryogenèse. Chez la souris transgénique, l'inactivation d'un allèle du VEGF est létale dès le huitième jour de la gestation [14]. Chez l'adulte, le VEGF est produit dans de nombreux tissus normaux, en particulier les reins, le cœur et le poumon. Le VEGF est sécrété par de nombreux types cellulaires dont les macrophages [15], les cellules péricytaires et les cellules épithéliales de la rétine mais pas par les cellules endothéliales elles-mêmes. Le VEGF est sécrété par les cellules cancéreuses. Ce peptide est surexprimé dans de nombreux cancers humains par rapport aux tissus de contrôle homologues [16-18].

Les fonctions du VEGF sont multiples :
- croissance et prolifération des cellules endothéliales (agent mitogène) ;
- différenciation et organisation des cellules endothéliales en tube (agent morphogène) ;
- augmentation de la perméabilité vasculaire (100 à 1 000 fois plus puissant que l'histamine ou la bradykinine) ;
- sécrétion et activation d'enzymes protéolytiques (métalloprotéases, plasminogène) provoquant la digestion de la matrice extracellulaire, qui facilitent l'infiltration des tissus par de nouveaux vaisseaux sanguins *(figure 1)*.

D'autres facteurs angiogéniques, les VEGF-B, VEGF-C et VEGF-D, ont été récemment clonés et possèdent de nombreuses homologies avec le VEGF. Ces facteurs constituent ainsi une famille de peptides angiogéniques [19-21].

Récepteurs du VEGF et de ses peptides apparentés

Les récepteurs du VEGF et de ses peptides apparentés appartiennent à la famille des récepteurs possédant une activité tyrosine kinase intrinsèque [22, 23]. Ces récepteurs, présents à la surface des cellules endothéliales, possèdent une région extracellulaire, composée de sept domaines de type immunoglobuline capable de lier les ligands, un domaine transmembranaire et un domaine cytoplasmique portant l'activité tyrosine kinase. La liaison du ligand provoque la dimérisation du récepteur et déclenche l'activité catalytique conduisant à la phosphorylation des récepteurs et de substrats cytoplasmiques. Trois récepteurs du VEGF ont été caractérisés :
- KDR chez l'homme *(kinase insert domain containing receptor* ; VEGF-R2) et Flk1 chez la souris *(fetal liver kinase-1)* ;
- Flt-1 *(fms-like tyrosine kinase-1* ; VEGF-R1) ;
- VEGF 165-R/neuropiline.

L'expression de Flt1 et Flk1, qui lient le VEGF avec une forte affinité, est restreinte aux cellules endothéliales et à certaines cellules hématopoïétiques monocytaires. Flk1/KDR intervient dans la vasculogenèse et dans l'angiogenèse, régulant la différenciation, le chimiotactisme et la multiplication des cellules endothéliales [24]. L'inactivation des deux allèles de ce gène est létale pour l'embryon de souris au neuvième jour de gestation [25]. Flt1 joue un rôle essentiel dans la différenciation et l'organisation architecturale des vaisseaux, sans posséder les activités mitogéniques et chimiotactiques contrôlées par Flk1 [22]. Ainsi le VEGF agit de façon paracrine via ses récepteurs pour réguler la différenciation des cellules endothéliales et la néovascularisation des tissus [26]. Un autre récepteur, Flt-4 *(fms-like tyrosine kinase-4)* présente de fortes homologies avec Flk1 et Flt1. Il lie avec une haute affinité les VEGF-C et VEGF-D et activerait la prolifération et la différenciation des cellules endothéliales lymphatiques [27].

La cascade de signalisation, activée par les récepteurs du VEGF et de ses peptides apparentés, commence à être mieux connue. Jusqu'à présent, les partenaires moléculaires de ces récepteurs sont communs à plusieurs tyrosines kinases et comprennent les molécules adaptatrices appartenant aux voies de transduction de SRC/RAS/MAP kinases, et comprenant la phospholipase C (PLCγ1) [28].

Autres facteurs angiogéniques

Le processus angiogénique ne peut être réduit à l'expression des peptides apparentés au VEGF et à l'activation de leurs récepteurs, mais il implique d'autres facteurs et d'autres familles de récepteur à activité tyrosine kinase. Ainsi, les récepteurs Tie-1 (récepteur orphelin) et Tie-2/Tek sont spécifiquement exprimés par cellules endothéliales et interviendraient dans l'établissement de l'architecture vasculaire [2]. Deux ligands de Tie-2 ont été identifiés : l'angiopoïétine-1 et l'angiopoïétine-2. L'angiopoïétine-1, sécrétée par les cellules mésenchymateuses, stimulerait le relargage du facteur de croissance dérivé des plaquettes (PDGF) par les cellules endothéliales. En retour, le PDGF recruterait les cellules mésenchymateuses. Leur interaction avec les cellules endothéliales conduirait à l'activation du TGF-β, présent sous une

forme latente dans la matrice extracellulaire, et à la différenciation des cellules mésenchymateuses en péricytes. L'angiopoïétine-1 serait donc responsable du maintien de l'intégrité des vaisseaux, tandis que l'angiopoïétine-2 seule induirait leur régression, et combinée au VEGF favoriserait le processus angiogénique. L'angiopoïétine-2 se comporte donc comme un antagoniste du récepteur Tie-2. D'autres facteurs de croissance ont un large spectre d'action, incluant l'angiogenèse. Le facteur de croissance des fibroblastes de type basique (bFGF) est un puissant mitogène pour les cellules endothéliales. Il agit de manière synergique avec le VEGF, et ce consécutivement à l'activation de son récepteur sur les cellules endothéliales, mais également en stimulant la sécrétion du VEGF par les cellules mésenchymateuses. Il convient de souligner que le bFGF existe essentiellement sous une forme inactive, associé aux protéoglycanes à héparane sulfate. La sécrétion de la protéine de liaison du bFGF par les cellules tumorales (FGF-BP) restaure la biodisponibilité et l'activité du facteur de croissance. Le facteur de croissance des hépatocytes/facteur de dissémination (HGF/*scatter factor*) a une action pléiotrope sur la prolifération, la motilité et la morphogenèse dans différents types cellulaires, incluant les cellules endothéliales [29].

ÉVALUATION DE L'ANGIOGENÈSE TUMORALE ET VALEUR PRONOSTIQUE

De nombreuses études ont démontré la valeur pronostique de l'angiogenèse tumorale appréciée par la mesure de la densité en microvaisseaux [30]. La mesure de la densité en microvaisseaux, par des techniques immunohistochimiques, a été appliquée dans de nombreux cancers solides. La densité en microvaisseaux est un facteur pronostique indépendant de la rechute locale ou métastatique dans le cancer du sein. Dans ce cancer, 21 études rétrospectives ont évalué la valeur pronostique de la densité en microvaisseaux. La grande majorité de ces études montre une association significative entre l'activité angiogénique élevée et un mauvais pronostic. Cette relation a été trouvée aussi bien pour les patientes avec ou sans ganglion métastatique [31, 32]. Dans les cancers urogénitaux [33], les cancers du poumon [34] et dans les cancers du tube digestif [35-39], la densité en microvaisseaux est un facteur pronostique de rechute essentiellement métastatique. L'expression du VEGF, mise en évidence par immunohistochimie, a fait l'objet d'études pronostiques rétrospectives dans le cancer du sein [32], le cancer de l'estomac [37] et le cancer du côlon [40, 41]. Dans le cancer du sein, c'est un facteur pronostique de rechute. Dans les cancers de l'estomac et du côlon, les résultats sont contradictoires [37-39, 40, 41]. Cependant, il convient de souligner que la quantification de l'angiogenèse en immunohistochimie est peu reproductible et difficile à standardiser du fait :
– de l'hétérogénéité de la vascularisation au sein d'une tumeur [41], une section tumorale donnée n'étant pas représentative de la vascularisation de l'ensemble de la tumeur ;
– de la diversité des anticorps monoclonaux utilisés (anticorps anti-facteur Willebrand, CD31 et CD34 pour la densité en microvaisseaux) ;
– de la faible différence d'expression des facteurs angiogéniques et de leurs récepteurs existant entre le tissu tumoral et le tissu adjacent de contrôle (différence rarement supérieure à un facteur 2).

Pour ces raisons, la densité en microvaisseaux et le VEGF ne sont pas des facteurs pronostiques utilisés en pratique courante. Une standardisation des méthodes de quantification de l'angiogenèse est cependant à l'étude [42], afin de valider l'intérêt pronostique de l'angiogenèse tumorale dans des études prospectives et multicentriques.

Peu de résultats sont disponibles sur la quantification de l'expression du VEGF évaluée en northern blot. Cette technique met en évidence l'accumulation d'ARN messagers dans les tumeurs et peut être standardisée vis-à-vis d'un autre messager, dont l'accumulation n'est pas modifiée pendant la progression néoplasique. L'expression relative de ces deux ARN messagers est quantifiée après hybridation des sondes radiomarquées (northern blot). Il existe une surexpression de VEGF d'un facteur 5 à 7 dans les cancers du rein et du sein [17, 18]. Cette différence est beaucoup plus faible dans le cancer du côlon chez l'homme [43]. En effet, nos résultats [43] montrent une très faible variation de l'expression des éléments de l'angiogenèse tumorale (VEGF, ses peptides apparentés et leurs récepteurs). Ce constat peut s'expliquer par l'importante vascularisation de l'épithélium digestif. Cependant, il ne faut pas négliger l'importance fonctionnelle de ce processus dans la croissance des tumeurs primaires et dans leur capacité à organiser l'invasion locale ou le processus métastatique. Dans le cancer du sein, la surexpression des messagers du VEGF [16, 17], notamment l'isoforme 121, est corrélée à un mauvais pronostic en analyse univariée. Dans le cancer du rein, modèle de tumeur hypervascularisée, la densité en microvaisseaux et l'expression du VEGF sont très élevées, notamment par rapport aux autres tumeurs solides [44, 45]. Actuellement, la quantification ou la mise en évidence in situ du VEGF, de ses récepteurs ou d'autres facteurs angiogéniques par les techniques de biologie moléculaire sont trop fastidieuses et trop onéreuses pour être utilisées dans la pratique médicale quotidienne. Plus que leur utilisation pronostique, ce serait un moyen de sélec-

tionner les types de tumeurs dont le profil serait approprié à l'application de thérapeutiques anti-angiogéniques spécifiques.

La validation de certaines protéases dans le pronostic des tumeurs a fait l'objet de nombreuses études. La cathepsine D est une protéase intervenant dans la dégradation de la matrice extracellulaire. Dans le cancer du sein, la cathepsine D a fait l'objet d'études pronostiques prospectives. Une absence de corrélation avec la taille et le grade est généralement rapportée. La majorité des études utilisant le dosage immunoradiométrique confirme que des taux élevés de cathepsine D sont associés à une survie sans métastase et à une survie globale plus courte [46, 47]. Ce facteur n'intervient pas actuellement dans les décisions thérapeutiques.

En conclusion, l'intérêt en clinique de ces facteurs pronostiques est controversé en raison du manque d'études prospectives et des limitations inhérentes à la quantification et à la standardisation des études immunohistochimiques.

L'ANGIOGENÈSE TUMORALE : UNE NOUVELLE CIBLE THÉRAPEUTIQUE ANTICANCÉREUSE

Le développement dans la cellule cancéreuse de mécanismes de résistances aux agents cytotoxiques est la principale cause d'échec des chimiothérapies (résistances intrinsèques ou acquises). La potentialité des cellules cancéreuses à échapper aux traitements cytotoxiques résulte notamment de leur instabilité génique (translocation chromosomique, amplification génique) qui conduit à la sélection de sous-population de cellules tumorales réfractaires à la chimiothérapie. Il apparaît qu'une stratégie thérapeutique, ciblant les cellules endothéliales (cellules normales diploïdes), pourrait permettre d'assurer le contrôle de l'évolution de la maladie. Les thérapeutiques anti-angiogéniques dirigées contre les cellules endothéliales n'induisent en principe pas ou peu de résistance [48, 49].

Une meilleure connaissance des mécanismes régissant l'angiogenèse tumorale devrait, à terme, permettre d'envisager de nouvelles perspectives thérapeutiques antitumorales par le blocage de la formation des vaisseaux irriguant les tumeurs [50-52]. De nombreuses équipes travaillent actuellement à l'élaboration d'agents anti-angiogéniques. Une dizaine d'agents sont actuellement testés dans des essais cliniques de phases I à III et beaucoup d'autres sont en développement *(tableau II)*. L'angiostatine et l'endostatine sont sur une voie de recherche très prometteuse, mais ces deux inhibiteurs de l'angiogenèse n'ont pas, à notre connaissance, été testés chez l'homme. Les composés anti-angiogéniques développés jusqu'à présent visent trois cibles associées aux cellules endothéliales que nous allons considérer successivement.

Tableau II. Agents anti-angiogéniques en cours d'évaluation dans les essais cliniques.

Phase I et II
Pentosan polysulfate
Analogues de la fumagiline : TNP-470 (AGM-470)
Platelet factor-4 (PF4)
Tecogalan (toxine de streptocoques du groupe B)
Thalidomide
Inhibiteurs des métalloprotéinases
Batimastat® (BB-94)
Marimastat® (BB-2516)
Néovastat® (inhibiteur dérivé du cartilage)
CM101 (exotoxine polysaccharidique du streptocoque)
Interleukine 12 (IL-12)
Anticorps neutralisant le VEGF
Inhibiteurs de l'activité tyrosine kinase des récepteurs du VEGF
Interférons
Suramine et analogues
Vitaxin
Phase III
Inhibiteurs des métalloprotéinases
Marimastat® (BB-2516)
Analogues de la fumagiline : TNP-470 (AGM-470)

Inhibition de la liaison des facteurs angiogéniques à leurs récepteurs au niveau des cellules endothéliales

La voie du VEGF constitue une cible majeure car de nombreuses approches consistant à contrôler le VEGF ou ses récepteurs (anticorps anti-VEGF ou antirécepteurs, ARN antisens) ont montré des effets antitumoraux in vivo, sur des modèles animaux [53-55]. Millauer et al ont montré, chez la souris athymique, que l'infection de cellules endothéliales par un rétrovirus codant le récepteur Flt1 muté (récepteur dominant négatif) était capable de bloquer la signalisation du VEGF et de prévenir ainsi la croissance de glioblastomes [56]. Des antagonistes du VEGF, notamment des substances capables d'inhiber l'activité kinase du récepteur Flk1 et des anticorps dirigés contre les récepteurs du VEGF et couplés à des toxines, sont actuellement en développement.

Interactions entre les cellules endothéliales et la matrice extracellulaire

Cette stratégie est basée sur les inhibiteurs des métalloprotéinases et les analogues de l'héparine qui inhibent la fixation des facteurs angiogéniques. Des études de phases I, II et III sont en cours [50, 57]. Les

métalloprotéinases sont impliquées dans la dégradation de la matrice extracellulaire. Plusieurs inhibiteurs de ces protéines sont en développement. Cette stratégie repose sur l'inhibition de la dégradation de la matrice extracellulaire pour empêcher l'invasion et la migration des cellules endothéliales et bloquer ainsi l'angiogenèse. Le Marimastat® (BB-2516) fait l'objet d'essais cliniques dans les cancers du pancréas, du poumon, de l'estomac et de l'ovaire. Le Néovastat®, extrait du cartilage de requin, inhibe l'angiogenèse [57]. Les analogues de l'héparine, tels que la suramine, le técogalan (dérivé d'un polysaccharide bactérien) et le pentosan sont des compétiteurs de l'héparine pour la fixation des facteurs angiogéniques (bFGF) et empêchent les cellules endothéliales de rompre la membrane basale et de migrer vers la tumeur. La suramine inhibe l'angiogenèse par des mécanismes multiples, dont la modulation de l'effet de stéroïdes ou de l'expression de molécules d'adhésion, ainsi qué la régulation négative de l'expression des récepteurs du bFGF. Le pentosan polysulfate a été testé dans les sarcomes de Kaposi associés au sida en phase I, il s'est révélé toxique et sans effet antitumoral significatif.

Transmission intracellulaire du signal et autres modes d'action

Le TNP 470 (AGM-1470), analogue synthétique d'un angiostatique fongique, la fumagiline, possède une activité cytostatique dirigée contre les cellules endothéliales. Il se fixe de façon covalente sur la méthionine aminopeptidase et inhibe son activité. C'est un inhibiteur spécifique de la prolifération des cellules endothéliales, de leur migration et de la formation des tubes capillaires [58, 59]. La fumagiline, produit par *Aspergillus fungus*, a été identifiée consécutivement à l'observation par l'équipe de Judah Folkman à Boston de l'inhibition de la croissance de cultures de cellules endothéliales contaminées par *Aspergillus fungus*. Le TNP-470 a été très étudié in vitro et in vivo. Il inhibe le développement de métastases dans des modèles de xénogreffes humaines et murines. Plusieurs essais sont en cours avec ce composé dans le cancer du pancréas, dont une phase II (traitement adjuvant après résection chirurgicale) et une phase III (TNP 470 versus association radio-chimiothérapie pour les tumeurs localement avancées).

L'interféron alpha-2a a été un des premiers agents identifiés pour exercer une activité anti-angiogénique, en faisant régresser les hémangiomes de l'enfant en inhibant la production du FGF [60, 61]. De même, la thalidomide, hypnotique utilisé dans les années 50 et responsable d'embryopathies, possède une activité anti-angiogénique, par antagonisme avec le TNF-α. Des études cliniques administrant la thalidomide per os chez des patients porteurs de divers carcinomes, sont en cours.

Il a été montré que les anticorps anti-intégrines $\alpha v\beta 3$ et $\alpha v\beta 5$ provoquent l'apoptose sélective des cellules endothéliales activées, car ces intégrines sont exprimées spécifiquement par les cellules épithéliales en prolifération [62]. Le vitaxin (anti-intégrine $\alpha v\beta 3$) fait l'objet d'essais de phase I. D'autres approches consisteraient à vectoriser des adénovirus recombinants pour un gène suicide, la thymidylate kinase de l'Herpès simplex virus, dont le gène serait placé sous le contrôle d'un promoteur spécifiquement activé dans la cellule endothéliale. Ce vecteur permettrait donc d'induire sélectivement la mort cellulaire dans le compartiment endothélial mobilisé par la néoangiogenèse tumorale, en mettant à profit le fait que ce compartiment se caractérise par la surexpression ou par l'induction de récepteurs angiogéniques peu représentés ou absents dans les cellules endothéliales normales. Un deuxième niveau de protection serait assuré par le fait que la cellule endothéliale normale ne se divise pas, contrairement au système mobilisé pendant l'angiogenèse tumorale. En effet, le ganciclovir, administré sous forme de prodrogue, serait donc converti en son métabolite ganciclovir-triphosphate et exercerait ainsi son effet cytotoxique, sélectivement dans les cellules endothéliales en division qui se dirigent vers la tumeur.

L'angiostatine et l'endostatine

La découverte de deux molécules anti-angiogéniques, l'angiostatine et l'endostatine, par l'équipe de Judah Folkman, ouvre de nouvelles perspectives thérapeutiques. Actuellement, la plupart des médicaments anti-angiogéniques en cours de d'expérimentation chez l'homme inhibent la prolifération et le développement de nouveaux vaisseaux, mais ils sont peu actifs sur des vaisseaux déjà établis. Les inhibiteurs des récepteurs du VEGF ou les inhibiteurs des métalloprotéinases provoquent chez l'animal un ralentissement de la croissance tumorale sans régression tumorale.

L'angiostatine, protéine de 38 kDa, fragment du plasminogène, est un inhibiteur spécifique de la prolifération des cellules endothéliales. Ce peptide est présent dans le sérum de souris athymiques quand la tumeur primitive xénogreffée est en place (carcinomes pulmonaires de Lewis). L'angiostatine n'est plus détectable dans la circulation 5 jours après l'exérèse de la tumeur primitive. Les métastases « à l'état de dormance », qui étaient incapables d'induire l'angiogenèse en présence de la tumeur primitive, deviennent subitement néovascularisées et entreprennent une croissance rapide après exérèse de la tumeur primitive [63]. Un tel mécanisme permettrait de rendre compte

des observations cliniques de poussées évolutives sur un mode métastatique consécutivement à l'exérèse de la tumeur primitive. De plus, l'administration d'angiostatine à des souris porteuses de tumeurs humaines maintient ces tumeurs dans un état latent. L'effet principal de l'inhibition de l'angiogenèse sur la croissance des tumeurs serait lié à une augmentation de l'incidence de l'apoptose des cellules tumorales [63].

L'endostatine a initialement été identifiée dans le milieu conditionné d'un hémangioendothéliome murin, pour sa capacité à inhiber la prolifération des cellules endothéliales en culture primaire [64]. C'est un fragment C terminal (20 kDa) du collagène XVIII, qui est lui-même un composant de la matrice extracellulaire. L'endostatine inhibe la croissance et permet la régression de différents modèles tumoraux (carcinomes pulmonaires de Lewis, fibrosarcome T241, mélanomes B16 et F10 et hémangioendothéliome E0MA) implantés chez la souris athymique [64]. Dans ces modèles, 5 à 14 jours après l'arrêt du traitement par l'endostatine, on assiste à une reprise de la croissance tumorale. Cependant, une série de deux à six cycles de traitement permet une régression des tumeurs sans développement de résistance et sans progression à l'arrêt du traitement. Les tumeurs restent « dormantes » pour une durée supérieure à 3 mois [49]. La plupart des cellules d'une tumeur traitée par l'endostatine meurent par un phénomène d'apoptose et ou de nécrose induite par l'hypoxie et le manque d'apport en facteurs de survie [49, 64]. L'endostatine, après administration, se dépose au niveau de la matrice extracellulaire, et les réinjections successives augmentent progressivement et durablement sa concentration dans la matrice, permettant l'inhibition de l'angiogenèse [65, 66].

L'angiostatine et l'endostatine sont moins susceptibles d'induire des résistances que les inhibiteurs ciblant les médiateurs de l'angiogenèse dérivés ou sécrétés par les cellules tumorales, qui peuvent induire une angiogenèse en sécrétant d'autres facteurs de croissance [48]. Ces résultats ont fait l'objet de nombreux commentaires et d'articles dans la presse, ayant donné des espoirs à de nombreux patients atteints de cancer. À notre connaissance, l'angiostatine et l'endostatine ne sont pas disponibles actuellement pour une expérimentation chez l'homme. Bien que la découverte de ces deux inhibiteurs ouvrent de nouvelles perspectives, une certaine prudence s'impose car les expériences menées chez la souris ne sont, en aucun cas, une garantie de succès chez l'homme.

DISCUSSION

Les produits anti-angiogéniques sont peu susceptibles d'éradiquer les tumeurs ou d'induire leur régression, mais ils peuvent inhiber ou ralentir la croissance tumorale et maintenir les micrométastases à l'état de dormance. Ainsi, dans les études cliniques à venir, la diminution de la masse tumorale ne sera probablement pas le bon critère d'évaluation. L'angiogenèse demeure un phénomène relativement restreint chez l'adulte sain (cicatrisation, cycle menstruel) et son inhibition sélective ne devrait pas entraîner d'effet secondaire majeur.

Les études chez l'animal montrent que les combinaisons entre plusieurs produits anti-angiogéniques sont plus efficaces qu'une monothérapie. De plus, la combinaison d'agents anti-angiogéniques et de produits cytotoxiques classiques augmente l'activité de ces derniers [59]. Les nouveaux agents anti-angiogéniques devront probablement être utilisés en combinaison avec des cytotoxiques mais également en monothérapie pendant de très longues durées afin d'inhiber la croissance tumorale. Les agents anti-angiogéniques ont montré une efficacité plus marquée sur les petites tumeurs, rendant leur administration possible en traitement adjuvant pour maintenir les métastases dans un état de dormance.

Certains médicaments anti-angiogéniques sont disponibles pour des essais thérapeutiques. Afin d'orienter les essais thérapeutiques utilisant des inhibiteurs de l'angiogenèse, les recherches futures devront identifier des marqueurs spécifiques susceptibles d'être la cible d'agents thérapeutiques ou constituer des facteurs pronostiques d'efficacité de ces nouvelles thérapeutiques. Ainsi, déterminer dans les tissus, dans le sang ou dans les urines, le niveau de production des facteurs angiogéniques pourrait permettre d'identifier les mécanismes prédominants de l'activation de l'angiogenèse pour chaque type de tumeur considéré, ce qui permettrait de constituer des critères objectifs d'indication de ces nouvelles thérapeutiques.

CONCLUSION

La recherche sur l'angiogenèse tumorale illustre bien le lien étroit existant entre l'analyse cognitive des mécanismes génétiques, moléculaires et cellulaires présidant à la croissance des tumeurs et à la définition de nouvelles hypothèses conduisant à de nouvelles alternatives thérapeutiques. Des études de phase I à III sont en cours afin d'évaluer la tolérance et l'efficacité antitumorale d'inhibiteurs de l'angiogenèse. Ces médicaments sont le plus souvent des cytostatiques non cytotoxiques. De nouvelles méthodologies d'évaluation sont nécessaires pour évaluer et optimiser leur action antitumorale. Des synergies sont possibles, en ciblant simultanément l'endothélium et la cellule cancéreuse, ouvrant ainsi des perspectives d'associations entre les agents cytotoxiques et les agents susceptibles d'éradiquer le compartiment endothélial des tumeurs

solides. L'angiostatine et l'endostatine sont deux inhibiteurs de l'angiogenèse très prometteurs, dont l'expérimentation n'a pas encore commencé chez l'homme.

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Articles

Inhibition of Arterial Thrombosis by Recombinant Annexin V in a Rabbit Carotid Artery Injury Model

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▸ Abstract

▲ Top
▪ Abstract
▼ Introduction
▼ Methods
▼ Results
▼ Discussion
▼ References

Background The procoagulant effect of anionic phospholipid may play a major role in the development of arterial thrombosis.

Methods and Results Annexin V, a calcium-dependent anionic-phospholipid–binding protein, was expressed and isolated from *Escherichia coli* and its antithrombotic effect examined in a rabbit carotid artery thrombosis model. A partially occlusive thrombus was formed in the left carotid artery by application of electric current to produce an ≈50% occlusion of the lumen. After the current was discontinued, flow ceased completely within 42±12 minutes (n=6) because of continuing platelet/fibrin thrombus formation. When annexin V was given at doses of 2.8 to 16.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for a period of 180 minutes, starting at the time the current was stopped, there was a dose-dependent inhibition of thrombus formation. At a dose of 5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$, blood flow remained patent throughout the infusion and for an additional 60 minutes after the infusion was stopped. In addition, there was a decrease in thrombus weight (16±7.4 versus 2.0±1.0 g), ^{125}I-fibrin deposition (≈45% reduction, $P<.001$), and ^{111}In-labeled platelet accumulation (≈43% reduction, $P<.001$). Prior mixing of annexin V with phosphatidylserine micelles abolished the antithrombotic effect of annexin V, whereas mixing with phosphatidylcholine micelles had no effect. The antithrombotic effect of annexin V was not associated with bleeding tendency, as judged by the amount of blood absorbed in a gauze pad placed in a surgical incision extending to the muscle tissue and by the standard template bleeding time.

Conclusions These observations support a potentially important role for anionic phospholipid exposure

in platelets in arterial thrombosis, and inhibition of this activity could be a novel target for therapy in coronary thrombosis and stroke and after angioplasty.

Key Words: annexins • thrombosis • phospholipids • hemodynamics

Introduction

▲ Top
▲ Abstract
• Introduction
▼ Methods
▼ Results
▼ Discussion
▼ References

Thrombosis of arteries plays a key role in the pathogenesis of a variety of ischemic syndromes, including unstable angina, myocardial infarction, and stroke. Arterial thrombus formation usually involves elements of vessel wall damage and platelet activation and aggregation. In addition to forming occlusive aggregates, activated platelets provide a highly efficient catalytic surface for the activation of prothrombin and factor X. The precise components of these catalytic surfaces are not fully elucidated, but anionic phospholipid constitutes a major component for binding sites for proteases and cofactors.[1,2] In platelets and in most other cells examined, anionic phospholipids are present only in the inner leaflet of the lipid bilayer membrane.[3] After the activation of platelets by certain agonists, anionic phospholipids move from the inner to the outer leaflet of the lipid bilayer; this rearrangement of membrane phospholipid is thought to be a major mechanism responsible for platelet procoagulant activity.[4,5,6] In addition, cultured endothelial cells have also been shown to provide a phospholipid-dependent procoagulant surface in vitro.[7,8] Furthermore, cells undergoing apoptosis have been shown to expose anionic phospholipid on their surfaces.[9] Thus, the exposure at the sites of vascular injury of anionic phospholipids from activated platelets and other cells may play a major role in the initiation, growth, and extension of the thrombus and may provide a novel target for antithrombotic therapy.

The annexins are a family of calcium-dependent anionic-phospholipid–binding proteins.[10] A member of this family, annexin V, was originally isolated from placenta, characterized as placental anticoagulant protein-I, and sequenced.[11,12] The same protein was also isolated and sequenced under different names.[13,14] Annexin V binds anionic phospholipids with a very high affinity in a calcium-dependent manner.[15] We have shown previously that annexin V binds to anionic phospholipid on platelets and blocks the binding of factors Xa and Va to platelets.[16,17] In addition, annexin V has been shown to be an effective inhibitor of thrombus formation in a venous thrombus model and in vitro perfusion models.[18,19,20] In the present study, we examined the potential role of annexin V as an inhibitor of arterial thrombosis in a carotid artery injury model developed in our laboratory. Our results indicate that intravenous infusion of annexin V can significantly inhibit thrombosis in this model without impairing the hemostatic response, even at doses that are three times greater than that required to inhibit thrombus formation.

Methods

▲ Top
▲ Abstract

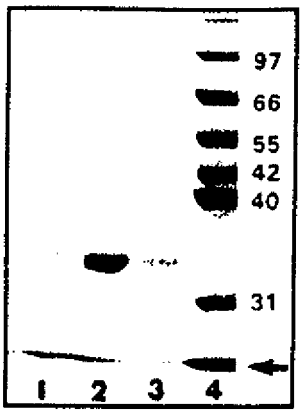
Preparation of Human Recombinant Annexin V

The polymerase chain reaction was used to amplify the cDNA from the initiator methionine to the stop codon with specific oligonucleotide primers from a human placental cDNA library (Clontec). The forward primer was 5'ACCTGAGTAGTCGCCATGGCACAGGTTCTC-3' and the reverse primer was 5'-CCCGAATTCACGTTAGTCATCTTCTCCACAGAGCAG-3'. The amplified 1.1-kb fragment was digested with *Nco* I and *Eco*RI and ligated into the prokaryotic expression vector pTRC 99A (Pharmacia Biotechnology Inc). The ligation product was used to transform competent *Escherichia coli* strain JM 105 and sequenced. The sequence of the amplified segment was identical to that published by Funakoshi et al.[12]

Recombinant annexin V was isolated from the bacterial lysates as described by Berger et al[21] with some modification. An overnight culture of *E. coli* JM 105 transformed with pTRC 99A–annexin V was expanded 50-fold in fresh Luria-Bertani medium containing 100 mg/L ampicillin. After 2 hours, isopropyl ß-D-thiogalactopyranoside was added to a final concentration of 1 mmol/L. After 16 hours of induction, the bacteria were pelleted at 3500*g* for 15 minutes at 4°C. The bacterial pellet was suspended in TBS, pH 7.5, containing 1 mmol/L PMSF, 5 mmol/L EDTA, and 6 mol/L urea. The bacterial suspension was sonicated with an ultrasonic probe (model W185, Heat System-Ultrasonic, Inc) at a setting of 6 on ice for 3 minutes. The lysate was centrifuged at 10 000*g* for 15 minutes, and the supernatant was dialyzed twice against 50 vol TBS containing 1 mmol/L EDTA and once against 50 vol TBS.

Multilamellar liposomes were prepared according to the method of Kinsky.[22] PS (Sigma Chemical Co), lyophilized bovine brain extract, cholesterol, and diacetylphosphate were dissolved in chloroform in a molar ratio of 10:15:1 and dried in a stream of nitrogen in a conical flask. TBS (5 mL) was added to the flask and agitated vigorously in a vortex mixer for 1 minute. The liposomes were washed by centrifugation at 3500*g* for 15 minutes, then incubated with the bacterial extract, and calcium chloride was added to a final concentration of 5 mmol/L. After 15 minutes of incubation at 37°C, the liposomes were sedimented by centrifugation at 10 000*g* for 10 minutes, and the bound annexin V was eluted with 10 mmol/L EDTA. The eluted annexin V was concentrated by Amicon ultrafiltration and loaded onto a Sephacryl S 200 column (5x90 cm). The annexin V was recovered in the included volume, whereas most of the liposomes were in the void volume. Fractions containing annexin V were pooled and dialyzed in 10 mmol/L Tris and 2 mmol/L EDTA, pH 8.1, loaded onto an anion exchange column (Resource Q, Pharmacia Biotechnology Inc), and eluted with a linear gradient of 0 to 200 mmol/L NaCl in the same buffer. The purified preparation showed a single band in SDS-PAGE under reducing conditions (Fig 1 ⊡). For rabbit experiments, the annexin V was dialyzed against HBS (10 mmol/L HEPES, 0.15 mol/L NaCl, pH 7.4) and sterile filtered with 0.2-µm filters.

Figure 1. SDS-PAGE of annexin V. Lane 1, 5 µL bacterial lysate; lane 2, 5 µL liposome-purified annexin V; lane 3, fast protein liquid chromatography–purified annexin V (5 µg); and lane 4, molecular weight standards. Samples were electrophoresed under reducing condition in 12% gels and stained with Coomassie brilliant blue R.



Arrow shows position of tracking dye.

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Unilamellar micelles of PS were prepared as described before.[23] Purified phospholipids were obtained from Avanti Polar Phospholipids, Inc. These micelles contained 20 mol% diheptanoylphosphatidylcholine and 80% dioleylphosphatidylserine (PS micelles) or dipalmitoylphosphatidylcholine (PC micelles). Equal volumes of annexin V (1 mg/mL) and phospholipid micelles (0.5 mg/mL) were mixed immediately before administration to the animals. For radiolabeled studies, annexin V was labeled with [^{125}I]NaI by the Iodo-Gen method to a specific activity of ≈350 cpm/ng as described previously.[24]

Rabbit Model of Carotid Artery Thrombosis

Carotid artery thrombosis was induced with electric current as described previously.[25 26 27 28] Male New Zealand White rabbits weighing 3.2 to 3.6 kg were anesthetized with ketamine (15 mg/kg) and xylazine (15 mg/kg). The right femoral artery was cannulated for recording of arterial blood pressure with a microtransducer (Electromedics). The right marginal ear vein was cannulated for administration of fluids and drugs. The right femoral vein was cannulated for drawing blood samples. The left common carotid artery was exposed by a median longitudinal incision in the neck, and a 2.5-mm Doppler flow probe was placed on the carotid artery without constricting the vessel. Proximal to the Doppler flow probe, a 4-mm-long, 23-gauge stainless steel needle electrode was inserted into the lumen of the carotid artery with minimal trauma. This electrode was positioned within the lumen parallel to the vessel wall. The bleeding was arrested by a piece of gel foam (Upjohn), and the needle was stabilized by a "surround collar" sutured around the vessel, which did not narrow the artery. After instrumentation, a 30-minute control period was allowed. During this time, blood pressure, heart rate, mean and phasic carotid artery blood flow, and ECG were continuously monitored. After this control period, thrombus formation was initiated by 150 μA of anodal current applied to the needle electrode until a 50% increase in flow velocity was recorded by the Doppler flow probe. This corresponds to an ≈50% decrease in cross-sectional area due to thrombus formation in the lumen.[25] To assess the degree and the variability of carotid occlusion at this point, a separate group of 16 rabbits were similarly instrumented, and current was applied until the carotid artery flow velocity increased by 50%. At this time, current was stopped

and the carotid artery pressure was fixed at arterial pressure by buffered glutaraldehyde infused through a perfusion catheter that was placed in the left common carotid artery proximal to the site of thrombus formation. After fixation, the vessel was paraffin-embedded and sectioned at 0.5-mm intervals. Twelve sections were made from each vessel, starting at the site of needle insertion and moving distally. After the sections were stained, planimetry was used to calculate the mean percentage of vessel occlusion by thrombus from the section that was most narrowed by thrombus in each vessel.

In initial studies, 4 rabbits were infused with annexin V (5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ IV) for 180 minutes without thrombus formation to determine whether annexin V had any inherent hemodynamic effects. After it was determined that annexin V did not any have direct effects, thrombus formation studies were done. At the time of 50% increase in flow velocity (≈50% occlusion of the vessel), the current was discontinued and rabbits were randomly allocated into 1 of 11 different treatment groups (see the Table ⊞) as follows: group 1, excipient (HBS), n=6; group 2, annexin V 2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes, n=6; group 3, annexin V 4.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes, n=6; group 4, annexin V 5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes, n=6; group 5, a mixture of annexin V and PS micelles (5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ of annexin V+2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ PS micelle) for 180 minutes, n=7; group 6, PS micelles 2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes, n=7; group 7, a mixture of annexin V and PC micelles (5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ of annexin V+2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ PC micelle) for 180 minutes, n=6; group 8, PC micelles 2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes, n=4; group 9, heparin 35 U/kg as an IV bolus followed by 0.5 $U \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes, n=6; group 10, heparin 35 U/kg as an IV bolus followed by 0.25 $U \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes, n=5; and group 11, monoclonal antibody against rabbit platelet IIb/IIIa receptor (0.5 mg/kg IV; AZ-1; gift from Michael Ezekowitz, MD, Yale University Medical School, New Haven, Conn), n=7. Where indicated, animals also received ^{125}I-fibrinogen (3 μCi in 1 mL of saline IV) at the time the current was stopped or ^{111}In-labeled platelets as described below. Fibrinogen was purified and radioiodinated as described previously to a specific radioactivity of 130 μCi/mg.[24 29]

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Table 1. Effect of Annexin V, Mixture of Annexin V and Phospholipid Micelles, Heparin, and Monoclonal Antibody to Platelet IIb/IIIa Receptor on Vessel Occlusion in the Electric Current–Induced Carotid Injury Model

^{111}In-Labeling of Platelets

Before instrumentation of the animals, blood was collected to label platelets with ^{111}In-8-hydroxyquinoline (oxine) as described previously.[28 29 30] Blood (21 mL) was collected into 4 mL of acid citrate dextrose and prostacyclin (10 μg/25 mL of blood), mixed, and centrifuged at 125*g* for 20 minutes at room temperature. The platelet-rich plasma was centrifuged at 1100*g* for 5 minutes and the platelet-poor plasma removed. The platelet pellet was suspended in 300 μL of platelet-poor plasma for labeling. [^{111}In]$InCl_3$ (Amersham Corp) was prepared in 0.3 mol/L acetate buffer, pH 5.3, to which 50 μL of oxine in ethanol (1 mg/mL) was added. After 15 minutes, the reaction mixture was extracted twice with 2 mL of methylene chloride and dried, and the residue was dissolved in 30 μL of absolute ethanol. Approximately 80% to 95% of the original radioactivity was recovered. ^{111}In-oxine (250 to 300 μCi) was then added to platelet suspensions for 30 minutes at 37°C, the mixture was centrifuged at 1100*g* for

5 minutes to remove the supernatant plasma, and the platelet pellet was resuspended in 1 mL of autologous plasma. The labeling efficiency was in the range of 50% to 80%. The in vivo viability of labeled platelets was determined by calculating the percentage of administered radioactivity bound to circulating platelets at different time intervals. At 2, 5, 10, 30, 60, and 120 minutes after the administration of ^{111}In-labeled platelets, a 1-mL blood sample was collected, platelets were isolated as described above, and radioactivity was counted in both the platelet pellet and platelet-poor plasma. The percentage recovery of the radiolabel, calculated as described before,[29] was found to be 80% to 90% in the platelet pellet.

Determination of the Accumulation of ^{125}I-Fibrinogen/Fibrin and ^{111}In-Labeled Platelets

Deposition of ^{125}I-fibrinogen/fibrin into carotid vessel segments was quantified as previously described.[26 27 28] In animals infused with ^{125}I-fibrinogen, either at the time of vessel occlusion or 60 minutes after the infusion was stopped, the left and the right carotid arteries were carefully removed and freed of all the surrounding fibrous tissue (carotid sheath). Then the left carotid artery was weighed and divided into three 2-cm segments: just proximal to the needle electrode insertion site into the vessel lumen; the site of thrombus formation, which corresponded to the position of the needle; and distal to the thrombus. Each segment was weighed, radioactivity was determined, and the counts were normalized according to the weight of the segment. Then the right carotid artery was weighed and, if necessary, trimmed so that its weight was the same as that of the left carotid artery, and radioactivity was determined. Accumulated radioactivity in the left carotid artery segments was expressed as a ratio of that measured from the uninstrumented right carotid artery.

Radiolabeled platelets were reinjected into the rabbits when the current was discontinued. At the end of the study (60 minutes after total occlusion or when the vessel remained patent for 60 minutes after the infusion of drugs was stopped), the carotid artery was removed and the ^{111}In-labeled platelet accumulation ratio was determined as described for the ^{125}I-labeled fibrinogen/fibrin accumulation ratio.

Rabbit Bleeding Assays

We evaluated the homeostatic parameters by two different methods. Template bleeding times were measured with the Simplate device (Organon Teknika). Uniform incisions 10 mm long and 1 mm deep were made on the ventral surface of the rabbit's ear in such a way as to avoid the superficial veins. Blood was blotted with filter paper (Whatman No. 4) every 30 seconds, avoiding the incision. Bleeding time was defined as the interval between the time of incision until blood did not stain the paper.

The incisional bleeding assay was a modification of previously published methods.[19 20] A surgical incision 4 cm long and 1 cm deep was made in the anterior abdominal wall, which incised the first layer of the anterior abdominal wall muscles. A preweighed gauze pad was placed in the incision for 5 minutes, and the amount of blood absorbed into the gauze was weighed. Both of the bleeding assays were performed before administration of annexin V or heparin (baseline) and then 15, 60, 180, and 300 minutes after administration of the test samples.

Tissue and Plasma Concentrations of Annexin V

To examine the tissue distribution and in vivo clearance of annexin V, ^{125}I-labeled annexin V (1.5 μCi,

specific activity ≈350 cpm/ng) was given intravenously to rabbits after the current was stopped, and 1-mL venous blood samples were collected at various times (0 to 120 minutes) (n=3). At the end of the study, the animals were killed by exsanguination under anesthesia, and the amount of radioactivity was counted in liver, spleen, right kidney, left kidney, brain, left ventricle (heart), aorta, left lung, right lung, normal proximal uninvolved segment of the left common carotid artery, the thrombosed segment of the same vessel, and the carotid artery thrombus. The radioactivity was expressed as cpm/mg wet tissue wt.

Assessment of Coagulation and Bleeding Parameters With Annexin V Infusion

To determine the potential of annexin V to induce coagulation and bleeding abnormalities, four incremental doses of annexin V (2.8, 4.2, 8.3, and 16.7 $\mu g \cdot kg^{-1} \cdot min^{-1}$ IV) were given by continuous infusion for 60 minutes (n=4; each animal received all four doses in ascending order over a 4-hour period), and aPTT, bleeding time, and incisional bleeding were assessed at the end of each dose before the next higher dose was begun.

Statistical Analysis

Data were analyzed by one-way ANOVA. In each figure, in the text, and in the Table⊞, mean values±SD are shown.

Results

▲ Top
▲ Abstract
▲ Introduction
▲ Methods
• Results
▼ Discussion
▼ References

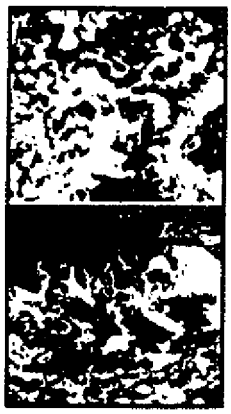
Hemodynamic Effects of Annexin V

Intravenous infusion of annexin V into the instrumented rabbit model, without the presence of thrombosis, for 180 minutes (5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$) (n=4) did not alter the blood pressure (pretreatment, 112±11 mm Hg versus 3 hours after infusion, 106±9 mm Hg), heart rate (pretreatment, 94±12 bpm versus 3 hours after infusion, 98±14 bpm), or carotid artery blood flow (pretreatment, 18.4±4.9 mL/min versus 3 hours after infusion, 19.2±3.8 mL/min). Thus, annexin V was devoid of any inherent hemodynamic effects detectable by these methods.

Time to Coronary Occlusion

Application of current to the left carotid artery for 47±21 minutes increased the flow velocity by ≈50%, as observed previously.[28] In the separate group of 16 animals, the examination of the carotid artery at this time point showed a partially occlusive thrombus that occupied 48.6±4% of the vessel lumen. The scanning electron microscopic appearance of the partially occlusive thrombus is shown in Fig 2A⊞, which demonstrates a predominantly platelet-covered surface for the formed thrombus and underlying fibrin scaffolding.[25,26] When the thrombus was removed, the underlying area of the carotid artery closest to the electrode showed endothelial removal and exposure of the subendothelial matrix (Fig 2B ⊞). When the current is discontinued at ≈50% occlusion, there is progressive thrombus growth with complete occlusion of blood flow within 42±12 minutes (Figs 3⊞, top, and 4A and Table⊞).

Figure 2. Top, Representative scanning electron micrograph of a thrombus from totally occluded artery. Lower arrow indicates platelet aggregates;



upper arrow indicates a red cell for comparison. Bottom, Representative scanning electron micrograph of artery at site of thrombus formation. Thrombus has been removed to show area of subendothelial exposure (lower arrow) and damaged endothelium adjoining area (upper arrow).

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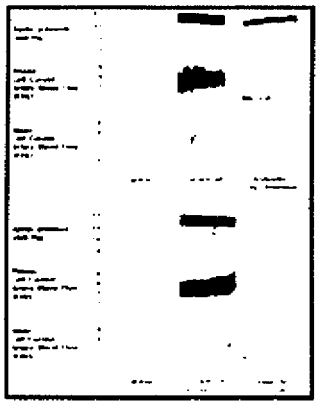

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Figure 3. Effect of annexin V infusion on carotid thrombosis in a rabbit model. Top, Control: baseline measurement of aortic pressure and phasic and mean carotid arterial blood flow; current off. Current application was stopped when a 50% increase in phasic and mean carotid blood flow velocities (corresponding to ≈50% decrease in cross-sectional luminal area) occurred. At this time, HBS was given. Occlusion by thrombus: 43 minutes after saline was given, there was total occlusion of blood flow. Bottom, Control and current off/annexin V depict same variables in an animal infused with annexin V 5.6 μg. Annexin V was given intravenously when current was stopped. Vessel did not occlude throughout study, and phasic wave form was preserved (similar to control).

When annexin V (5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was given, starting at the time when current was discontinued, blood flow remained unchanged throughout the infusion and then for an additional 60 minutes after the infusion was stopped (total duration of maintenance of blood flow, >240 minutes) (Figs 3⊞, bottom, and 4A and Table⊞). Note that the phasic carotid artery blood flow has a normal systolic and diastolic phasic blood flow profile at the end of 240 minutes (Fig 3B⊞). Because in the presence of severe narrowing (high-grade stenosis) the phasic flow pattern is gradually lost (flow is predominantly during systole), the preservation of the normal carotid flow pattern by annexin V indicates that there was no further narrowing of the vessel by ongoing thrombus formation. This observation is further supported by the fact that examination of the vessels from animals infused with annexin V revealed only small, nonocclusive thrombi containing fibrin and platelets compared with occlusive thrombi of the same

apparent histological composition in control animals infused with HBS (Fig 4B). Hemodynamic variables (arterial blood pressure, heart rate, and phasic and mean carotid blood flow) were not affected by the infusion of annexin V.

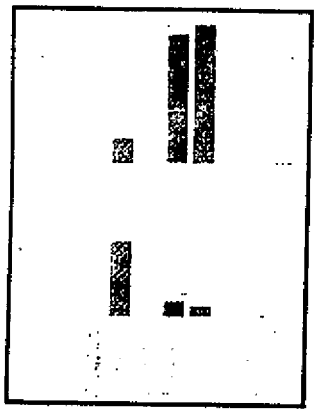

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Figure 4. Effect of infusion of HBS buffer (control), mixture of annexin V and PS micelles, PS micelles alone, different doses of annexin V, and heparin on thrombus formation in rabbit carotid thrombosis model. A, Duration of patency of vessel (time required for occlusion of vessel after current was stopped). B, Weight of residual thrombus in carotid artery at end of experiment. There is a dose-dependent effect of annexin V on vessel patency, with a reduction in thrombus weight. A similar effect was observed with high dose of heparin. *P<.05, **P<.001 vs control (HBS buffer) group.

Annexin V inhibited thrombus formation in a dose-dependent manner, being effective in all animals receiving 5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ but ineffective at a dose of 2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (Fig 4A and Table). When animals were anticoagulated with heparin (35 U/kg as an IV bolus followed by 0.5 $U \cdot kg^{-1} \cdot min^{-1}$), the left carotid artery also remained patent (5 of 6 animals), although lower levels of heparin (35 U/kg as an IV bolus followed by 0.25 $U \cdot kg^{-1} \cdot min^{-1}$) did not prevent carotid occlusion (Fig 4A and Table). Similarly, the administration of monoclonal antibody to rabbit IIb/IIIa receptor (0.5 mg/kg IV) prevented carotid artery thrombosis (5 of 7 animals) (Table). However, there was a significant reduction in platelet counts after the administration of the antibody (before, $264\pm32x10^5/mm^3$ versus 4 hours after, $92\pm18x10^5/mm^3$, P<.001; 4 hours after without antibody, $214\pm46x10^5/mm^3$, P=NS). After the administration of monoclonal antibody to IIb/IIIa, there was no significant increase in bleeding for the first 1 to 2 hours. After 3 to 4 hours, however, a marked increase in bleeding at incisional sites, as well as significant blood loss by the incisional method, was observed (data not shown). Lower doses of the monoclonal antibody were ineffective in preventing carotid artery thrombosis.

We investigated the effect of PS micelles on the antithrombotic activity of annexin V. When annexin V at a dose equivalent to 5.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes was mixed with PS micelle at a dose equivalent to 2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes and then infused, the effect of annexin V was markedly diminished (Fig 4A and Table). Infusion of the same dose of PS or PC micelles (2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 180 minutes) had no significant effect compared with animals receiving HBS (Fig 4A and Table).

Radiolabeled Fibrinogen Accumulation in Thrombus

Because annexin V would be expected to interfere primarily with the platelet procoagulant mechanism ultimately leading to the generation of fibrin, it was important to assess its effect on the deposition of radiolabeled fibrinogen/fibrin in the thrombosed carotid segment (Fig 5A⊞). Animals were infused with ^{125}I-fibrinogen at the time the current was stopped, and ^{125}I-fibrinogen/fibrin accumulation was measured. The accumulated fibrinogen/fibrin was expressed as the ratio of radioactivity in the segment from the instrumented artery to that of the radioactivity in the corresponding segment of the contralateral nonmanipulated artery. In the presence of annexin V, ^{125}I-fibrinogen/fibrin accumulation ratios decreased in the thrombosed segments by ≈47% (from 42±4.2 [untreated] to 22.1±4.7 [treated], P<.001), whereas there was no difference in the segment proximal or distal to the lesion.

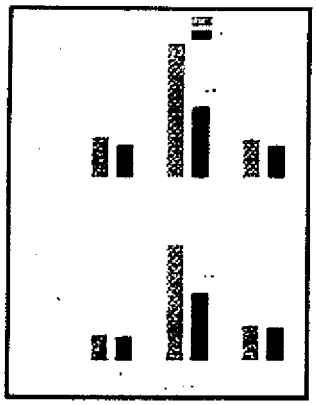

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Figure 5. Effect of annexin V infusion on deposition of ^{125}I-fibrinogen/fibrin in common carotid artery (A) and on accumulation of ^{111}In-labeled platelets in same vessel (B). A, After discontinuation of electrical stimulation of left common carotid artery, when a 50% increase in blood flow velocity was observed, ^{125}I-fibrinogen (3 μCi) was infused with (n=6, hatched bar) or without (n=6, solid bar) annexin V (4.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Animals infused with ^{125}I-fibrinogen alone were killed at time of total occlusion, and segments of both common carotid arteries were removed. Animals that also received annexin V did not have occluded left common carotid artery, and same vessel segments were obtained by killing this group at 240 minutes. ^{125}I-fibrinogen/fibrin accumulation ratios were then determined in proximal (Prox.), thrombosed (site of needle electrode insertion), and distal portions of left common carotid artery as described in "Methods." B, Experiment was performed as in A, except that ^{111}In-labeled platelets were infused instead of ^{125}I-fibrinogen. A and B, Values are mean±SD. $^{**}P$<.02 vs controls.

Platelet Accumulation in Thrombus

Because platelets are an important component of the thrombus in the electrically induced carotid thrombosis model and thrombin-induced platelet aggregation is thought to contribute significantly to this process, it was important to determine the effect of annexin V on platelet contribution to intravascular clotting in this setting (Fig 5B⊞). To compare platelet deposition in the thrombosed, proximal, and distal segments of the left carotid artery, a platelet accumulation index using ^{111}In-labeled platelets was used. The counts in the left and right carotid segments were compared as for labeled fibrinogen. In animals treated with annexin V, the platelet deposition indices decreased by ≈42% (from 76.1±6.9 [untreated] to 44.0±9.7 [treated], P<.001) in the thrombosed segment of the carotid artery. There were no significant changes in the platelet deposition indexes observed in the proximal or distal carotid artery segments. These data suggest that annexin V also caused a significant decrease in platelet deposition into the thrombus. To verify the viability of labeled platelets, we determined the ^{111}In counts in blood as a function of time after injection of labeled platelets into the rabbits. Two minutes after injection, 95% of the injected counts were present; at 5 minutes, 78%; at 10 minutes, 64%; and at 30 minutes, 62%. No significant decrease in ^{111}In activity was noted after 30 minutes (80% to 90% of this circulating

radioactivity was associated with the platelets). This strongly suggests that the damaged platelets are rapidly removed by spleen or other reticular-endothelial cells and that the remaining circulating ^{111}In was localized in normal platelets.

Clearance and Tissue Distribution of Annexin V

Clearance of annexin V was rapid, with an α-phase of $\approx$5 minutes, at the end of which 10% to 12% radioactivity remains in circulation (Fig 6A). The majority of the annexin V was cleared through the kidneys (data not shown). Among the vascular structures, the highest ratio of blood to tissue was found in the thrombus (Fig 6B). After the thrombus was removed, the thrombosed left common carotid artery segment containing the thrombus did not show a significantly higher count compared with other vascular structures, such as right carotid artery or aorta. We also infused ^{125}I-labeled annexin V starting at the time of initiation of current (initiation of thrombus formation) (n=3). The objective was to determine whether annexin V would bind to the carotid arterial wall if there was no thrombus to cover the area of injury. After 180 minutes of infusion, the left carotid artery was carefully removed and counted, and it did not show a significantly higher count compared with the uninstrumented right carotid artery or the aorta. These results indicate that annexin V concentrates preferentially in the thrombus and not in the vessel wall.

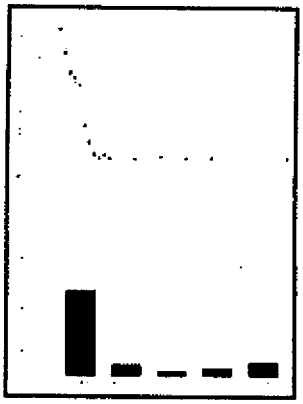

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Figure 6. Clearance of ^{125}I-labeled annexin V in rabbit blood after administration of 1.5 μCi ^{125}I-labeled annexin as intravenous (IV) infusion (A). Note rapid decrease in blood levels when infusion is stopped. B, Tissue distribution of ^{125}I-labeled annexin V at end of 180 minutes of IV infusion. Note high concentration in thrombus relative to vessel wall. LCA indicates left carotid artery; RCA, right carotid artery.

Alterations in Hemostatic and Bleeding Parameters

Infusion of annexin V results in the prolongation of aPTT in rabbit plasma (Fig 7). The control aPTT was 26.9±4.6 seconds; with 2.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ of annexin V infusion, it was 34.7±2.7 seconds; with 4.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 37.5±7.0 seconds; with 8.4 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 37.7±4.1 seconds; and with 16.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 36.0±3.3 seconds. Although the prolongation of aPTT was statistically significant ($P<.05$), the increasing doses of annexin V infusion did not cause a significant further prolongation in aPTT, unlike heparin infusion (27.4±2.2 seconds with 35 U/kg bolus+0.25 $U \cdot kg^{-1} \cdot min^{-1}$ IV and 55.0±8 seconds with 35 U/kg bolus+0.5 $U \cdot kg^{-1} \cdot min^{-1}$ IV) (Fig 7). The effect of annexin V on hemostatic

response to a cutaneous abdominal wound was assessed after a standardized incision by weighing the amount of blood absorbed by a sponge placed in the wound for 5 minutes (Fig 8A). Animals infused with either saline, annexin V, or a mixture of annexin V and PC or PS micelles did not bleed excessively compared with untreated controls. Consistent with this observation, when abdominal incision bleeding was measured, no difference in accumulated radioactivity in the gauze pad was noted between annexin V–treated and control animals that had been infused with ^{125}I-fibrinogen. In contrast, animals receiving heparin at levels required to prevent occlusive carotid thrombosis (0.5 U · kg^{-1} · min^{-1}) had markedly increased bleeding. The dose of heparin used was then steadily decreased until a level was reached at which there was only a moderate increase in bleeding from the abdominal wound (0.25 U · kg^{-1} · min^{-1}) (Fig 8A). At this concentration of heparin, however, occlusion of the left carotid artery occurred at time intervals similar to those noted in saline-treated control animals (Table). The contrast between the increased bleeding tendency of animals receiving heparin (35 U/kg bolus followed by 0.5 U · kg^{-1} · min^{-1}, the amount of heparin required to prevent formation of an occlusive thrombus) and the apparent lack of bleeding in animals treated with annexin V was also qualitatively evident throughout the experimental manipulations with regard to blood loss at incisions in the chest wall and catheter insertion sites. Neither annexin V and nor heparin produced a significant change in template bleeding times (Fig 8B).

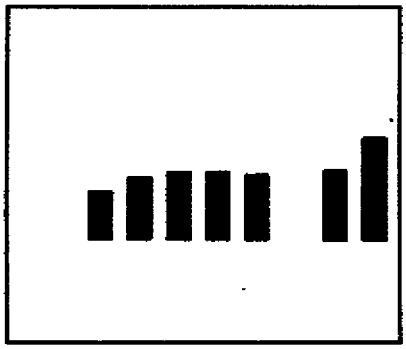

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Figure 7. Effect of annexin V or heparin infusion on aPTT of rabbit plasma. aPTT was performed on citrated plasma samples from animals infused with various doses of annexin V or heparin. *P<.001 vs control.

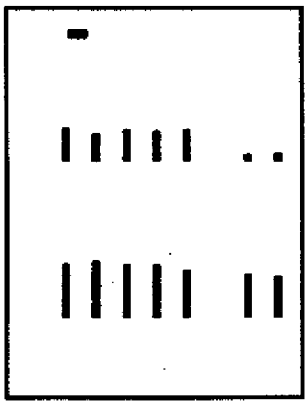

Figure 8. Effect of annexin V or heparin infusion on bleeding in response to a standardized abdominal incision (A) and template ear bleeding time (B). Amount of blood loss into a standardized abdominal incision (1x4 cm) was measured by a 4x4-in preweighed gauze pad placed into incision for 5 minutes, removed, and weighed to determine accumulated blood. Experimental groups received either annexin V or heparin at indicated dose, and blood loss before indicated treatment (solid bars) and 1 hour later (hatched bars) is shown. Template bleeding time was time required for spontaneous arrest of bleeding from an ear incision. Experimental groups were the same in A and B. Values are mean±SD. *P<.001 vs baseline.

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Discussion

▲ Top
▲ Abstract
▲ Introduction
▲ Methods
▲ Results
▪ Discussion
▼ References

After vessel wall injury, platelets rapidly adhere to the damaged vessel wall and to one another to form the primary hemostatic plug. In addition, after activation, platelets accelerate the generation of thrombin by providing an effective catalytic surface for the conversion of prothrombin by factor Xa (in the presence of factor VIIIa and Ca^{2+}) and factor X by factor IXa (in the presence of factor VIIIa and Ca^{2+}).[1 2 3] Both factor Va and factor VIIIa were shown to bind with high affinity to a limited number of binding sites in platelets, ≈1000 sites for factor Va and ≈400 sites for factor VIIIa.[31 32 33 34] The occupancy of Va receptor is essential for the subsequent binding of factor Xa, whereas binding of factor VIIIa, though not essential for factor IXa binding, enhances the binding affinity of IXa by fivefold.[33] Platelet-bound factor Xa catalyzes the activation of prothrombin 300 000 times (K_{cat}/K_m) faster than in solution, and platelet-bound factor IXa catalyzes the activation of factor X 17 000 000 times faster than in solution.[1 2 31 32 33 34] The nature of the binding site has not been fully elucidated, but anionic phospholipid is required for formation of this binding site.

The importance of the exposure of anionic phospholipid for hemostasis in vivo is demonstrated in Scott syndrome, a rare bleeding disorder associated with deficiency of platelet procoagulant activity.[35] The platelets in this disorder have decreased exposure of anionic phospholipid after platelet activation and have reduced factor Va-Xa and factor IXa binding sites.[36 37] Whether the exposure of anionic phospholipid is also important in the pathogenesis of thrombosis associated with vessel wall injury is not known. The expression of platelet anionic phospholipids in vitro requires a stronger stimulus than that for the induction of aggregation and secretion.[4 6 16 17] Such strong stimuli may be provided in vivo in arterial injury by the exposure of subendothelial components to the adhering platelets. Furthermore, the high fluid shear stress typically seen at these sites may be an additional stimulus, resulting in a strong activation. The exposure of anionic phospholipid by these stimuli may play a significant role in the growth of platelet-rich thrombi in arteries as their growth is stabilized by a fibrin scaffold.

Our results show that in a model of arterial thrombosis, anionic phospholipids play a significant role in thrombus formation. The intravenous infusion of annexin V significantly inhibited thrombus formation in a dose-dependent manner, as judged by maintenance of blood flow with normal systolic and diastolic phasic profiles and inhibition of fibrin deposition and platelet accumulation at the site of arterial injury. Most significantly, the prior mixing of annexin V with PS micelles attenuated the antithrombotic effect of annexin V, whereas prior mixing with PC micelles had no effect. These results suggest that mechanistically, the anticoagulant effect of annexin V is mediated by its interaction with PS, presumably on activated platelets. Annexin V has been shown to be selectively taken up in the thrombi in vivo.[38] It

is also possible that annexin V may have similar effects on endothelial or other cell types at the site of vascular injury.

One intriguing finding to emerge from these studies was the lack of hemostatic compromise observed in rabbits treated with annexin V, even at three times the concentration required to induce an antithrombotic effect. In contrast, the dose of heparin required to maintain vascular patency induced significant bleeding at extravascular sites. Although one must be careful not to overinterpret these results, if this finding is verified in other models of arterial thrombosis, it may provide important clues to the inherent differences between the mechanisms involved in hemostasis and those involved in thrombosis. The two methods used to evaluate hemostasis, the template bleeding time and the abdominal incision method, both evaluate bleeding from capillary sources. Occlusion by an arterial thrombus requires the continued recruitment and incorporation of platelets into the developing thrombus after the first wave of platelets has recognized the site of injury. These platelets are laid down on a scaffold of fibrin, which requires activation of a soluble coagulation system. In small capillaries, on the other hand, thrombus growth is much less crucial to achieve a thrombus mass sufficient to occlude the vessel. Thus, the procoagulant activity associated with platelets may not be required. Another possible explanation for the disparity between the antithrombotic effect and the antihemostatic effect of fibrin is the observation that annexin V does not inhibit the activity of the tissue factor VIIa complex as effectively as it inhibits prothrombinase activity.[39] In extravascular tissues, the tissue factor–initiated clotting mechanisms may be less amenable to inhibition. Finally, the differential effect can also be due to the most obvious differences between capillaries and arteries: the ratio of endothelial surface to blood volume. In capillaries, where this ratio is large, the altered endothelial surface may suffice to bring about the arrest of bleeding, whereas in large arteries, where this ratio is small, platelet procoagulant activity may have a predominant role. In any event, our data suggest that the platelet procoagulant activity is not as important for capillary hemostasis as for arterial thrombosis. Of interest, the Ms Scott for whom the Scott syndrome was named has a normal bleeding time.[35]

In conclusion, our experiments show that the inhibition of procoagulant activity of platelets by annexin V can attenuate experimentally induced arterial thrombosis in the absence of excessive bleeding. These observations lend support to the notion that anionic phospholipid exposure in platelets has an important role in arterial thrombosis. Inhibition of this activity may be a novel target for antithrombotic therapy in conditions associated with arterial injury, such as acute myocardial infarction and stroke, and after angioplasty.

Selected Abbreviations and Acronyms

aPTT = activated partial thromboplastin time
HBS = HEPES-buffered saline
PC = phosphatidylcholine
PS = phosphatidylserine

TBS = Tris-buffered saline

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Footnotes

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▲ Top
▲ Abstract
▲ Introduction
▲ Methods
▲ Results
▲ Discussion
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